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IN THE NAME OF

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for

Detection of Micro Metastasis of Melanoma and Breast Cancer in Paraffin-Embedded Tumor Draining Lymph Nodes by Multimarker Quantitative RT-PCR

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DETECTION OF MICRO METASTASIS OF MELANOMA AND BREAST CANCER IN PARAFFIN-EMBEDDED TUMOR DRAINING LYMPH NODES BY MULTIMARKER QUANTITATIVE RT-PCR

This invention claims priority to the U.S. Provisional Patent Application No. 60/426,216 filed November 14, 2002.

Background of the Invention

The present invention relates to methods of detection of micro metastasis in lymph nodes and methods of predicting the recurrence of the disease and survival. More specifically, the invention is directed to detection of micro metastasis in cancerous tissue and lymph nodes by using multimarker real-time reverse transcriptase polymerase chain reaction assay.

Description of the Prior Art

The incidence of malignant melanoma has been increasing in the United States over the past decade (Balch, C.M., 2001; Jemal, A.J., 2002; and Rigel, D.S., 1996). Breast cancer is another common form of malignant disease whose incidence is increasing. One of the most devastating aspects of cancer is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and develop into metastases. Despite advances in surgical treatment of primary neoplasms and aggressive therapies, most cancer patients die as a result of metastatic disease.

Cancers are staged according to a well-defined, elaborate progressive scale, developed by the American Joint Committee on Cancer (AJCC). Caught early, cancer is very often curable. The five-year survival rate varies considerably depending on the AJCC stage level. For example, for Stage I and Stage II melanoma, the five-year survival rate is over 80%. However, for Stage IV the survival rate is less that 20% (AJCC). Similarly, the overall five-year breast cancer

survival rate is about 75 percent for white women and about 63 percent for black women. This rate rises to nearly 90 percent for women with Stage I or II cancer (U.S. Pat. No. 6,057,105, which is incorporated herein by the reference). Therefore, accurate determination of an AJCC stage is extremely important for selecting patients for adjuvant therapies and for directing follow-up procedures.

Until recently, a complete dissection of regional lymph nodes was performed to obtain nodal staging information. All lymph nodes of the lymphatic drainage basin were removed for pathology examination for the presence of micrometastases (Balch, C.M., 1996; Cascinelli, N., 1998; Li, W., 1999). However, such approach is labor-intensive. Also, about 80% of patients who undergo elective lymph node dissection have no evidence of metastases and do not benefit from this procedure.

John Wayne Cancer Institute (JWCI) has pioneered an alternative sentinel lymphadenectomy (SLND) technique for the treatment of early stage melanoma (Morton, D.L., 1992; Morton, D.L., 1993; Morton, D.L., 1999). Sentinel lymph node (SLN) is defined as the first lymph node in the regional lymphatic basin that drains the primary tumor. Several reports have confirmed that the SLN is the first node that receives metastatic melanoma cells and that the SLN reflects the metastatic status of the entire lymphatic basin (Morton, D.L., 1992; Li, W., 1999). Conceptually, a tumor-negative SLN predicts the absence of tumor metastases in the other regional lymph nodes (non-SLN) with a high degree of accuracy. Typically, the probability of non-SLN in the draining lymphatic basin containing melanoma cells is less than 1% when the SLN does not have metastatic melanoma cells (Morton, D.L., 1992). Thus, in the case of a tumor-negative SLN, this method allows to avoid complete lymph node dissection and various postoperative complications associated with such procedure.

Standard procedure for the histopathologic examination of regional lymph nodes utilizes hematoxylin and eosin (H&E) staining. But this procedure often underestimates the presence of micrometastatic disease. Immunohistochemistry (IHC) staining with HMB-45 and S-100 antibodies can increase the sensitivity of detecting melanoma cells in lymph nodes tenfold over H&E staining alone (Bostick, P.J., 1999; Cochran, A.J., 1984; Shivers, S.C., 1998; Li, W., 1999).

Development of reverse transcriptase polymerase chain reaction (RT-PCR) assays and gene markers for different tumor types, such as tyrosinase for melanoma, made it possible to detect occult metastases in lymph nodes of cancer patients whose disease was not found by either H&E or IHC techniques (Li, W., 2000). Despite initially encouraging results, the clinical and survival significance of these occult metastases detected by RT-PCR assays has not been demonstrated conclusively. Furthermore, conventional single mRNA marker RT-PCR assays are limited in their ability to discriminate cancer cells from normal cells that also carry the marker and, thus, these methods suffer from reduced specificity and reliability. In addition, tumor heterogeneity has caused sensitivity problems where a single mRNA marker was employed – although all tumor cells within a primary tumor or metastasis may express the same marker gene, the level of specific mRNA expression can vary considerably. Thus, despite the identification of melanoma and breast cancer markers, these markers cannot individually detect tumor cells in a highly specific and sensitive manner (U.S. Patent No. 6,057,105).

A multimarker RT-PCR assay eliminates some of the problems associated with single-marker detection techniques. To date, several multiple-mRNA marker (MM) RT-PCR assays have been developed for detecting occult metastatic melanoma cells in blood, bone marrow, or lymph nodes (Taback, B., 2001; Sarantou, T., 1997; Bostick, P.J., 1999; Hoon, D.S.B., 2000; Miyashiro, I., 2001). For example, a marker combination of tyrosinase and melanoma-associated antigens MART-1 and MAGE-A3 have been used to detect occult melanoma cells in frozen sections of SLNs (Bostick, P.J., 1999). Although this study reported a great improvement of detection sensitivity compared to H&E staining and IHC, the study did not determine clinicopathologic importance of the detection. Also, since the study limited the follow-up period to 12 months and recurrence of melanoma, typically, takes 3-8 years, it failed to establish an ability of the selected markers to predict disease recurrence and patient's overall survival.

A panel of three tumor mRNA markers, beta-chain human chorionic gonadotropin (β-hCG), hepatocyte growth factor (C-Met), and universal MAGE (uMAGE) was used in a combination with RT-PCR to detect nodal micrometastases of colorectal cancer. But the study did not establish the prognostic significance of the method (Bilchik, A.J., 2000).

The U.S. Patent No. 6,057,105 describes an RT-PCR method of detecting metastatic melanoma cells with a set of melanoma marker genes, including tyrisonase, MART-1, tyrosinase related protein-1 (TRP-1), and MAGE-A3. The patent also describes an RT-PCR method of detecting breast cancer cells with a set of marker genes, including C-Met, glycosyltransferase β-1,4-N- acetylgalacto-saminyltransferase (GalNAcT), β-hCG, MAGE-A3, MAGE–2, and Cytokeratin –20 (CK20). Although the methods of the U.S. Patent No. 6,057,105 are more sensitive than RT-PCR with a single marker, they fail to predict a long-term (at least 3 years) disease recurrence and survival.

The U.S. Patent No. 6,037,129 describes an RT-PCR method of detecting metastatic breast cancer cells with a set of marker genes, including c-Myc, PIP, and keratin-19. This patent utilizes regression analysis to determine a correlation between number of positive RT-PCR markers and predicted survival per AJCC stage. However, similarly to the U.S. Patent No. 6,057,105, the U.S. Patent No. 6,037,129 does not conduct a retrospective long-term (greater than 5 years) analysis of the samples and, thus, does not evaluate prognostic significance of the selected markers. Also, the selected set of the markers has a limited utility as it produced a large number (40%) of false positive results.

In summary, none of the currently available methods provides a reliable prediction of disease recurrence, patient's prognosis and survival, particularly in AJCC stage I, II and III patients.

Summary of the Invention

In view of the described shortcomings of the existing methods for detection metastatic cancer cells, it is one object of the present invention to provide high sensitivity methods for detection of metastatic melanoma, breast cancer, gastric cancer, pancreas cancer, or colon cancer cells. It is another object of the present invention to provide a method for predicting disease recurrence within at least three-year period, preferably at least five-year period, following removal of the primary melanoma and SLND. It is also an object of the present invention to identify a panel of molecular markers that can detect micrometastasis in tumor draining lymph nodes (TDLN) and sentinel lymph node (SLN). It is also an object of the present

invention to provide methods for identification of metastasis in TDLN/SLN that are histopathologically negative as determined by H&E staining and IHC.

These and other objects are achieved in a method of detecting metastatic melanoma cells in a patient. The method comprises (a) isolating nucleic acid from a biological sample obtained from the patient; (b) amplifying nucleic acid targets, if present, from a panel of marker genes, wherein the panel comprises GalNAcT, a transcription factor PAX3, or both; and (c) detecting the presence or absence of the nucleic acid targets. The panel may further comprise markers selected from a group consisting of MAGE-A3, GalNAcT, MART-1, PAX3, MITF, TRP-2, and Tyrosinase.

In one embodiment the panel comprises MAGE-A3, GalNAcT, MART-1, and PAX3. In another embodiment the panel comprises MART-1, GalNAcT, MITF, and PAX3. In further embodiment the panel comprises MART-1, TRP-2, GalNAcT, and PAX3. In still another embodiment the panel comprises Tyrosinase, MART-1, GalNAcT, and PAX3. MART-1 is a melanoma antigen on melanosomes; GalNAcT is a glycosysltransferase for synthesis of gangliosides GM2 and GD2; Tyrosinase is a melanogenesis pathway enzyme to make melanin; Tyrosinase-related protein-2 (TRP-2) is a melanogenesis pathway enzyme. MITF and PAX3 are transcription factors.

In one embodiment, the panel of mRNA markers is used in a combination with RealTime RT-PCR (qRT-PCR) assay. The nucleic acid may be isolated from paraffin-embedded (PE) melanoma tissues, frozen lymph nodes, and PE lymph nodes. Prior to the present invention there had been no evidence in the art that the described panels of markers are useful for predicting disease outcome.

In another aspect, the present invention provides a method of detecting metastatic breast, gastric, pancreas or colon cancer cells in a patient. The method comprises (a) isolating nucleic acid from PE cancerous tissues or PE lymph nodes of the patient; (b) amplifying nucleic acid targets, if present, from a panel of marker genes selected from a group consisting of C-Met, MAGE-A3, Stanniocalcin-l, mammoglobin, heat shock protein 27 (HSP27), GalNAcT, cytokeratin 20 (CK20), and beta chain-human chorionic gonadotrophin (β-HCG); and (c) detecting the presence or absence of the nucleic acid targets. In one embodiment, the panel of mRNA markers is used in a combination with RealTime RT-PCR (qRT-PCR) assay.

Prior to the present invention there had been no evidence in the art that the described panels of markers when used to amplify nucleic acid isolated from PE cancerous tissues or PE lymph nodes are useful for predicting disease outcome.

The methods of the present invention are rapid, repeatable and quantitative. They were validated using 215 melanoma patients in retrospective analysis with greater than 5 years follow up, which is a minimum amount of follow up needed to verify the prognostic significance of disease outcome in early stage patients.

In a different aspect, the present invention provides a kit for use in detecting melanoma cells in a biological sample. The kit comprises pairs of primers for amplifying nucleic acids targets from a panel of marker genes, wherein the panel comprises GalNAcT, PAX3, or both, and containers for each of the pairs of primers.

The present invention provides a number of unexpected advantages. First, it allows to determine stages of the cancers more accurately as compared to the existing methods. The methods and kit of the present invention allow accurate determination of a need for further lymph node removal surgery and/or adjuvant treatment. Second, the present invention demonstrates the better reliability of molecular marker panels of the present invention in prediction of the disease outcome and recurrence as compared to conventional prognostic factors such as age, gender, Breslow thickness, Clark level, site, ulceration. The panels of the present invention provide an accurate prediction of disease recurrence in histopathology negative SLN ($p < 0.0001$) and survival ($p < 0.0001$). The methods of the present invention diagnose metastasis in SLN with high sensitivity and specificity.

The invention is defined in the appended claims and is described below in its preferred embodiments.

Description of the Figures

The above-mentioned and other features of this invention and the manner of obtaining them will become more apparent, and will be best understood by reference to the following description, taken in conjunction with the accompanying drawings. These drawings depict only a typical embodiment of the invention and do not therefore limit its scope. They serve to add specificity and detail.

Figure 1 shows representative quantitative RealTime RT-PCR (qRT-PCR) analysis for MART-1 mRNA copy levels. **Figure 1A** depicts signal increase of the

serially diluted plasmid containing MART-1 cDNA (10^1 to 10^8 copies) standard templates with the increase of the number of PCR cycles. RFU is relative Fluorescence unit. **Figure 1B** shows the standard curve (correlation coefficient 0.998) generated by using the threshold cycle (Ct) of templates with known numbers of copies. Symbol ∘ indicates standards.

Figure 2 provide receiver operating characteristic (ROC) curves for mRNA tumor markers.

Figure 3 shows the disease free survival (DFS) rate for patients with histopathologically positive and negative SLNs.

Figure 4A shows DFS rate for melanoma patients with histopathologically negative SLNs with no mRNA markers expressed and with one or more mRNA markers expressed in accordance with one embodiment of the present invention, in which of MART-1, MAGE-A3, GalNAcT, and PAX3 markers were utilized.

Figure 4B shows DFS rate for melanoma patients with histopathologically negative SLNs with no mRNA markers expressed and with one, two or three mRNA markers expressed in accordance with one embodiment of the present invention, in which of MART-1, MAGE-A3, GalNAcT, and PAX3 markers were utilized.

Figure 5 shows DFS rate in accordance with one embodiment of the present invention, in which of MART-1, MAGE-A3, GalNAcT, and PAX3 markers were utilized. The graph shows DFS rate for patients with histopathologically negative SLNs; DFS for patients with no mRNA markers expressed; and DFS for patients with one or more mRNA expressed.

Detailed Description of the Preferred Embodiments

The present invention provides highly sensitive, multimarker methods to detect melanoma, breast, gastric, pancreas or colon cancer cells in SLNs of patients with or without clinical evidence of disease. The methods of the present invention are designed to overcome limitations in existing technologies with respect to sensitivity, specificity, and ability to predict outcome of the disease.

Recent studies have indicated that patients with histopathologically melanoma-free SLNs, who are positive for two or more mRNA markers as determined by RT-PCR, were at a significantly increased risk of recurrence

compared with those who were positive for one or fewer mRNA markers (Bostick, P.J., 1999). However, because frozen tissue sections were used for these MM RT-PCR assays, a large number of SLN samples could not be assessed and large-scale retrospective analysis with a follow up of greater than three years could not be conducted. Accordingly, patient's survival under long term follow-up period could not be analyzed.

The instant invention solved this problem by utilizing paraffin-embedded (PE) specimens for MM RT-PCR assays to assess much larger patient population of SLN samples and analyze patient's longer follow-up survival data and to determine the significance of detection of molecular metastasis. One major advantage of the PE qRT-PCR assay is that retrospective analysis of specimens from multiple sources can be assessed and evaluated for clinicopathological utility.

Until recently, it was believed that mRNA cannot be preserved in PE tissues due to degeneration. It was also believed to be difficult to isolate mRNA from PE tissues for PCR analysis (Masuda, N., 1999; Shirota, Y., 2001). However, some recent investigations have proved the utility of RT-PCR assays using mRNA isolated from PE specimens (Masuda, N., 1999; Shirota, Y., 2001; Specht, K., 2000; U.S. Pat. No. 6,248,535; U.S. Pat. No. 6,602,670, all of which are incorporated herein by the reference). Additionally, the use of PE samples facilitates correlation of histopathological and RT-PCR data. This is because most of histopathological samples are prepared as formalin fixed and paraffin. These samples are retained in archival storage of a large number of pathology departments, along with their clinical histories and prognoses (U.S. Pat. No. 6,248,535; Masuda, N., 1999). Frozen sections are rarely, if ever, used for histopathology analysis. Additionally, frozen sections are logistically difficult to obtain and assess by RT-PCR.

In addition, recent development of RealTime RT-PCR (qRT-PCR) assay allows the rapid and reproducible quantitative analysis with high sensitivity and specificity. It is a high-throughput technology based on an online fluorescence detection system that allows sensitive detection and quantitation of gene expression (Shirota, Y., 2001). But even with these significant developments in the art, inability to predict recurrence of cancer after removal of the primary tumor remains to be a major problem.

In summary, the multi-marker panels of prior art, such as those of the U.S.Pat. No. 6,057,105, do not have a significant predictive power in disease recurrence. The marker panels of prior art that were created for frozen sections would not be predictive in PE samples. Earlier studies had at the most a twenty-month follow-up (Li, W., 2000) and could not evaluate the predictive power of markers for disease recurrence, which takes 3-8 years. Additionally, many of the markers utilized in earlier MM RT-PCRs were producing false-positive results (Li, W., 2000, U.S. Patent No. 6,037,129).

The present invention solves these problems by providing a method of detecting metastatic melanoma cells in a patient. The method comprises (a) isolating nucleic acid from a biological sample of the patient; (b) amplifying nucleic acid targets, if present, from a panel of marker genes, wherein the panel comprises GalNAcT, PAX3, or both; and (c) detecting the presence or absence of the nucleic acid targets. The biological sample may be selected from a group consisting of PE melanoma tissues, frozen lymph nodes, and PE lymph nodes. The biological sample may be histopathologically negative or positive for melanoma cells. Typically, the histopathology of the biological sample is determined by hematoxylin and eosin staining or immunohistochemistry. However, other suitable methods can also be used.

The present invention unexpectedly demonstrates that GalNAcT and PAX3 are new promising molecular marker for detecting occult melanoma cells. By using a large well-defined patient population with a significant clinical follow up, the present invention unexpectedly demonstrated that the quantitative detection of these mRNA markers in SLNs in the patients with early-stage melanoma has clinicopathological and prognostic utilities.

GalNAcT is a key enzyme in the biosynthetic pathway of gangliosides GM2/GD2, which are oncofetal glycolipids found elevated in expression on the surface of melanomas (Hoon, D.S., 1991; Tai, T., 1983). In melanoma, gangliosides GM2 and GD2 expression, are often enhanced to very high levels and associated with tumor progression including metastatic tumors. Gangliosides are also highly expressed in breast cancer cells. The gangliosides GM2 and GD2 are immunogenic in humans and can be used as a target for specific immunotherapy such as human monoclonal antibodies or cancer vaccines (U.S. Pat. No. 6,057,105).

PAX3 transcription factor has been reported to regulate melanin synthetic pathway via MITF expression (Goding, C.R., 2000). PAX3 is well expressed in human melanomas and contributes to melanoma cell survival (Goding, C.R., 2000; Scholl, F.A., 2001). Recent studies have suggested the clinical usefulness of PAX3 gene expression as specific markers for detecting melanoma cells (Scholl, F.A., 2001).

In another embodiment of the present invention, the panel of markers may further comprise markers selected from a group consisting of MAGE-A3, GalNAcT, MART-1, PAX3, MITF, TRP-2, and Tyrosinase. MART-1 and MAGE-A3 are major melanocyte differentiation antigens that are immunogenic in patients and well expressed in melanoma (Kawakami, Y., 1994; Kawakami, Y., 1994; Marincola, F.M., 2000; Schultz, E.S., 2000). MAGE-A3 has high specificity and expression in melanoma cells (Sarantou, T., 1997; Miyashiro, I., 2001). Clinical utilities of assessing MAGE-A3 mRNA expression in primary tumor, metastatic lesion, and blood in melanoma patients have been previously reported (Miyashiro, I., 2001). Tyrosinase is a melanogenesis pathway enzyme to make melanin; Tyrosinase-related protein-2 (TRP-2) is a melanogenesis pathway enzyme. MITF is a transcription factor.

In one embodiment the panel comprises MAGE-A3, GalNAcT, MART-1, and PAX3. In another embodiment the panel comprises MART-1, GalNAcT, MITF, and PAX3. In further embodiment the panel comprises MART-1, TRP-2, GalNAcT, and PAX3. In still another embodiment the panel comprises Tyrosinase, MART-1, GalNAcT, and PAX3.

In one embodiment, mRNA expression levels of MART-1, MAGE-A3, GalNAcT, and PAX3 in PE SLNs were analyzed retrospectively using a qRT-PCR assay in 215 patients with early-stage melanoma. The study demonstrated the reliability of qRT-PCR assays with the selected panel of markers and using PE tissues of the large number (n = 215) of patients with at least 5yr follow-up after SLND. Previous research by the inventors has revealed that patients with histopathologically melanoma-free SLNs who were MM RT-PCR positive were at significantly increased risk of recurrence compared with those who were negative for mRNA markers (Bostick, P.J., 1999). However, the previous studies using frozen SLNs did not demonstrate the clinical usefulness of the assays and the

selected marker sets because of the limitation of the number of patients and a shorter follow-up period after SLND.

The four mRNA markers of this embodiment are specific for and frequently found in melanomas (Sarantou, T., 1997; Miyashiro, I., 2001; Kawakami, Y., 1994; Kawakami, Y., 1994; Marincola, F.M., 2000; Schultz, E.S., 2000; Hoon, D.S., 1991; Tai, T., 1983; Kuo, C.T., 1998; Goding, C.R., 2000; Scholl, F.A., 2001). All four of the mRNA markers were expressed in 100% of melanoma cell lines. However, the mRNA copy number for individual markers vary in individual cell lines. Also, the mRNA copy levels in histopathology positive PE SLNs are not expressed in 100% but vary in individual SLNs. The results are due to not only the heterogeneity in individual tumor tissues but also the portion of micrometastases which have completely removed from the PE-block in some cases at the time of pathological examination (H&E and IHC), and also the quantity of the amount of mRNA for each sample.

In this embodiment of the present invention, at least one positive marker was identified in 89% of histopathologically positive SLNs demonstrating the accuracy of this study. MAGE-A3 alone was positive in 45% of positive SLNs. This result is compatible with a previous study in which MAGE-A3 mRNA was expressed in 43% of metastatic melanoma (Miyashiro, I., 2001). GalNAcT mRNA was expressed in 64% of histopathology positive SLNs. This result is consistent with earlier reported study (Kuo, C.T., 1998). PAX3 mRNA expression was identified from 77% of primary cultured melanoma but not identified from melanocyte using *in situ* hybridization (Scholl, F.A., 2001).

Among 162 histopathology negative SLN patients, 48 (30%) patients were upstaged by the (multi-marker panel (MM) qRT-PCR assay of the present invention. Interestingly, the increased number of expressed markers significantly correlated with worse disease-free survival (DFS) after SLND. By contrast, DFS and survival rate for the patients with no expression of any of the markers of the panel of the present invention was significantly high. Recurrence of the disease in patients who had no MM expression in SLNs may be due to the false-negative results by qRT-PCR assay or spread of disease through hematogeneous metastasis. In fact, eight (67%) of 12 patients who had no MM expression but recurred had hematogenic

metastases as an initial recurrence site and the other four patients had lymphatic metastases at least 2yr later after SLND.

The present invention demonstrate that when marker panels of the present invention are used, lack of expression of markers can predict favorable prognosis of the patients who had SLND. Interestingly, the mRNA copy number of each marker in patients who recurred was significantly higher than that in patients who did not recur among the histopathology negative SLN patients.

The qRT-PCR assays of the present invention not only provided qualitative but also quantitative data in predicting the patient's survival. Among the histopathology negative SLN patients, occult melanoma cells with higher MM copy levels correlate with disease recurrence. The present invention also demonstrates that histopathology negative and RT-PCR negative patients will remain disease-free and need less management.

The qRT-PCR assays of the present invention provides a rapid and reproducible approach with high sensitivity and specificity. Moreover, the assay does not require tedious and often subjective procedures required in detection of PCR products, such as in gel electrophoresis- based assays. The present invention demonstrates feasibility and utility of the MM qRT-PCR assays in PE samples by ROC curve analysis, comparison study between frozen and PE specimens of the same specimen, and dilution analysis using laser capture microdissection (LCM).

Accordingly, in one embodiment of the present invention, the panel of mRNA markers is used in a combination with qRT-PCR assay. It is a discovery of the present invention that the use of qRT-PCR in a combination with the panel of markers of the present invention greatly improves assay sensitivity and specificity for the detection of occult metastases in PE SLNs, which are H&E and IHC negative, and predict disease outcome in patients with early stage melanoma.

Accordingly, in one embodiment, the method for detecting metastatic cells of the present invention comprises a step of assigning an AJCC (American Joint Committee on Cancer) stage to the patient based on the presence or absence of the nucleic acid targets in the sample. In another embodiment, the method for detecting metastatic cellscomprises a step of predicting at least one parameter selected from a group consisting of disease recurrence, patient's prognosis, and patient's survival, wherein the parameters are determined based on the presence or absence of the

nucleic acid targets in the sample. Preferably, the parameter is predicted for at least three-year period following a removal of a primary tumor, sentinel lymphadenectomy (SLND), or both. The method may further comprise a step of selecting a treatment regimen based on the patient's prognosis.

It is also a discovery of the present invention that quantitative multiple marker expression in the PE SLN may more accurately reflect occult metastases of melanoma and be a more powerful predictor of patients' disease relapse and postoperative survival than H&E and IHC. Prior to the present invention there had been no evidence in the art that the described panels of markers are useful for predicting disease outcome. Accordingly, in another aspect, the present invention provides a method for detecting metastatic melanoma cells in PE samples. The method comprises (a) deparaffinizing PE melanoma tissue samples or PE lymph node samples to obtain deparaffinized samples; (b) isolating nucleic acid from the deparaffinized samples; (c) amplifying nucleic acid targets, if present, from a panel of marker genes, wherein the panel comprises at least two marker genes selected from a group consisting of MAGE-A3, GalNAcT, MART-1, PAX3, MITF, TRP-2, and Tyrosinase; and (d) detecting the presence or absence of the nucleic acid targets.

In another aspect, the present invention provides a method of detecting metastatic breast, gastric, pancreas or colon cancer cells in a patient. The method comprises (a) isolating nucleic acid from PE cancerous tissues or PE lymph nodes of the patient; (b) amplifying nucleic acid targets, if present, from a panel of marker genes selected from a group consisting of C-Met, MAGE-A3, Stanniocalcin-l, mammoglobin, heat shock protein 27 (HSP27), GalNAcT, cytokeratin 20 (CK20), and beta chain-human chorionic gonadotrophin (β-HCG); and (c) detecting the presence or absence of the nucleic acid targets.

C-Met is a cell surface receptor for hepatocyte growth factor (motility, growth, migration activation). MAGE-A3 is of unknown function. Characterized as an immunogenic antigen in human found in different cancers not normal tissue except testes and placenta. Sanniocalcin-1 is of unknown function in humans. Found in animals and fish to regulate calcium and phosphate levels. Mammoglobulin is related to secretions of the mammary gland. HSP 27 is a regulator of various cellular functions including apoptosis and is activated in malignancy. Cytokeratin 20 is

cytoskeleton protein regulating shape and motility. Beta-HCG is a gonadotropin hormone that binds to beta-HCG receptor and is involved in growth and metabolic activation of a cell.

In one embodiment, the panel of mRNA markers is used in a combination with RealTime RT-PCR (qRT-PCR) assay. Prior to the present invention there had been no evidence in the art that the described panels of markers when used to amplify nucleic acid isolated from PE cancerous tissues or PE lymph nodes are useful for predicting disease outcome.

Accordingly, in one embodiment, the method for detecting metastatic breast, gastric, pancreas or colon cancer cells of the present invention comprises a step of assigning an AJCC (American Joint Committee on Cancer) stage to the patient based on the presence or absence of the nucleic acid targets in the sample. In another embodiment, the method for detecting metastatic cellscomprises a step of predicting at least one parameter selected from a group consisting of disease recurrence, patient's prognosis, and patient's survival, wherein the parameters are determined based on the presence or absence of the nucleic acid targets in the sample. Preferably, the parameter is predicted for at least three-year period following a removal of a primary tumor, sentinel lymphadenectomy (SLND), or both. The method may further comprise a step of selecting a treatment regimen based on the patient's prognosis.

In a different aspect, the present invention provides kits for use in detecting melanoma cells in a biological sample. All the basic essential materials and reagents required for detecting melanoma cells in a biological sample, may be assembled together in a kit. This will generally comprise of the preselected primers for two, or more, particular specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Tag, etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

Such kits will generally comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair. In one embodiment, the kit comprises primers for amplifying nucleic acids targets from a panel of marker genes, wherein the panel comprises GalNAcT, PAX3, or both, and containers for each of the pairs of primers. In another embodiment, the kit further

comprises primers from marker genes selected from a group consisting of MAGE-A3, MART-1, MITF, TRP-2, and Tyrosinase.

Kits of the present invention, also will typically include a means for containing the reagents in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired reagent are retained. Other containers suitable for conducting certain steps of the disclosed methods also may be provided.

The foregoing is meant to illustrate, but not to limit, the scope of the invention. Indeed, those of ordinary skill in the art can readily envision and produce further embodiments, based on the teachings herein, without undue experimentation.

Example 1

Patients and Tumors

SLN specimens were obtained in consultation with the surgeon and pathologist at the John Wayne Cancer Institute (JWCI). Informed human subject IRB consent was obtained from patients for the use of all specimens. All patients had AJCC stage I or II malignant melanoma (staged by current AJCC staging system)(Balch, C.M., 2001) which are defined as those patients with no clinical evidence of regional or metastatic disease. SLND was performed in the patients as previously described (Morton, D.L., 1992). Total 308 SNs obtained from 215 patients were assessed for histopathologic examination and qRT-PCR assay. The patients ranged in age from 18.7 to 91.3 years old (mean age 50.9 ± 15.9 SD), consisting of 127 males and 88 females. The patients who had histopathologically (H&E/IHC) proven metastatic melanoma in SLNs at SLND subsequently received complete lymph node dissection of the lymphatic basin. Patients were followed up by clinical diagnostic examinations in the outpatient clinic. The patients who had melanoma recurrence after SLND received a variety of adjuvant therapies (immunotherapy, chemotherapy, or biochemotherapy)(Morton, D.L., 1992; Morton, D.L., 1996; O'Day, S.J., 2002). The personnel performing the qRT-PCR assay did not know the disease outcome of patients. The JWCI melanoma computer database analysis with patients' follow-up and history was independently provided to a biostatistician.

Thirty-two pathology-verified metastatic lesions from melanoma patients undergoing elective surgeries at JWCI were used for positive controls and for

assessing receiver operating characteristic (ROC) curves (Henderson, A.R., 1993; Mitas, M., 2001). After the patients provided informed consent, 39 pathology-defined cancer negative lymph nodes obtained from patients undergoing tonsilectomy, colorectomy or breast surgery were utilized as negative controls and for assessing ROC curves.

Ten melanoma cell lines (MA, MB, MC, MD, ME, MF, MG, MH, MI, and MJ) established and characterized at the John Wayne Cancer Institute (JWCI) were used as positive controls for the qRT-PCR assay. All established cell lines were grown in RPMI-1640 medium supplemented with 100 ml/L heat-inactivated fetal calf serum, penicillin, and streptomycin (GIBCO, Grand Island, NY) in T75-cm^2 flask as described previously (Miyashiro, I., 2001). Total RNA was extracted from cells when cell cultures reached 70-80% confluence.

Example 2

Histopathologic Examination and RNA Isolation

Histopathologic examination by the pathologists was performed on each collected SN as previously described (Bostick, P.J., 1999, which is incorporated herin by the reference). Each SN was bisected, and an 8-μm imprint slide was then prepared from the tissue surface and stained with Diff-Quik I & II (Dade International, Miami, FL) for the pathologist's intraoperative diagnosis. If melanoma cells were identified in the frozen section, a complete lymph node dissection was then performed (Bostick, P.J., 1999). Six immediately adjacent frozen sections were cut on the cryostat to a thickness of 12 μm each and stored at –80°C until processed at a later date (Bostick, P.J., 1999; Miyashiro, I., 2001). The remainder of the bisected node, which was not frozen, was then placed in 10% formalin and embedded in paraffin, and 4-μm-thick sections were examined with H&E staining. Adjacent 4-μm-thick sections werre evaluated by IHC using antibodies to HMB-45 and S-100 proteins. This PE section evaluation was performed at two different levels separated by approximately 40 μm.

For qRT-PCR assay, additional 10 sections of 10-μm thick tissues were cut from each tissue specimen embedded in paraffin using a microtome and new sterile blade. The sections were placed in a sterile container for deparaffinization with xylene. Deparaffinized tissue sections were then subjected to proteinase K digestion and RNA extraction using the Paraffin Block RNA Isolation Kit (Ambion, Austin,

Texas). Briefly, tissues were digested, RNA was solubilized in a guanidinium-based buffer, the separation of RNA was achieved by using phenol:chloroform, and isopropanol was used to precipitate the RNA. Pellet Paint (Novagen, Madison, WI) was also used in the precipitation procedure to enhance the recovery of RNA. The RNA was dissolved in molecular grade water and quantified using a spectrophotometer and RIBOGreen detection assay (Molecular Probes, OR). Total cellular RNA from cell lines and frozen tissue specimens was extracted, isolated and purified using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (Sarantou, T., 1997; Bilchik, A.J., 2001). All RNA extractions were performed in a designated sterile laminar flow hood using RNAse-free lab ware. Tissue processing, RNA extraction, RT-PCR assay set-up, and post-RT-PCR product analysis were performed in separate designated rooms to prevent cross-contamination, as previously reported (Bostick, P.J., 1999).

Example 3

Dilution Study

To verify the specificity of detecting occult metastasis, total RNA was extracted from 1000 melanoma cells of obviously metastatic melanoma tissue (n = 8) in PE sections using laser capture microdissection (LCM). Five-μm thick sections were cut from each tissue specimen embedded in paraffin using a microtomb and mounted on a slide. Then, tissue sections were deparaffinized with xylene and subsequently stained with H&E. After drying a slide, 1000 metastatic melanoma cells were microdissected using the PixCell II LCM System (Arcturus Engineering, Mountain View, CA) as manufacture's procedure. Microdissected tissues were digested with proteinase K and total RNA was extracted using the Paraffin Block RNA Isolation Kit (Ambion). RNA isolated from 1000 melanoma cells was dissolved in molecular grade water, and was serially diluted by 1/2, 1/10, 1/20, 1/50, 1/100, 1/500, and 1/1000. Reverse transcriptase reaction and qRT-PCR assays were performed for each marker on the diluted RNAs.

Example 4

Primers and Probes

Primer and probe sequences were designed for the RealTime PCR assay using Oligo Primer Analysis Software, version 5.0 (National Biomedical systems, Plymouth, MN). To avoid possible amplification of contaminating genomic DNA,

primers were designed so that each PCR product covered at least one intron. Fluorescence Resonance Energy Transfer (FRET) probe sequences were as follows:

MART-1: 5'-FAM-CAG AAC AGT CAC CAC CAC CTT ATT-BHQ-1-3' (SEQ ID NO:1);

MAGE-A3: 5'-FAM-AGC TCC TGC CCA CAC TCC CGC CTG T-BHQ-1-3' (SEQ ID NO:2);

GalNAcT: 5'-CAL RED-ATG AGG CTG CTT TCA CTA TCC GCA-BHQ-2-3' (SEQ ID NO:3);

PAX3, 5'-FAM-CCA GAC TGA TTA CGC GCT CTC CC-BHQ-1-3'(SEQ ID NO:4);

Glyceraldehydes-3-phosphate dehydrogenase (GAPDH): 5'-FAM-CAG CAA TGC CTC CTG CAC CAC CAA-BHQ-1-3' (SEQ ID NO:5).

Control melanomas and non-melanoma tissues and cell lines were used to optimize the assay. GAPDH was utilized as internal reference house-keeping genes for status of sample mRNA assessed.

Additionally, the following probes may be used:

MITF: 5'-FAM-AGA GCA CTG GCC AAA GAG AGG CA-BHQ-1-3' (SEQ ID NO:6).

TRP-2: 5'-FAM-TCA CAT CAA GGA CCT GCA TTT GTT A-BHQ-1-3'(SEQ ID NO:7).

Tyrosinase (TYR): 5'-FAM-TTC ACC ATG CAT TTG TTG ACA GTA TT-BHQ-1-3' (SEQ ID NO:8).

C-MET: 5'-FAM-TGG GAG CTG ATG ACA AGA GGA G-BHQ-1-3' (SEQ ID NO:9).

Stanniocalcin-1: 5'-FAM-CCT GCT GGA ATG TGA TGA AGA CAC-BHQ-1-3'(SEQ ID NO:10).

Mammoglobin: Sense: 5'-CACTGCTACGCAGGCTCT –3' (SEQ ID NO:11); Antisense: 5'-TGCTCAGAGTTTCATCCG –3' (SEQ ID NO:12).

HSP27: 5'-FAM-AGG AGC GGC AGG ACG AGC AT-BHQ-1-3'(SEQ ID NO:13).

Cytokeratin 20: 5'-FAM-ATC AGT TAA GCA CCC TGG AAG AGA G-BHQ-1-3'(SEQ ID NO:14).

Beta-HCG: 5'-FAM-CCT GCC TCA GGT GGT GTG CAA C-BHQ-1-3'(SEQ ID NO:15).

The above-listed probes are examples of probes that may be used. Those skilled in the art will recognize that the sequences of markers of the present invention are known and other primers for the markers of the present invention can be easily synthesized.

Example 5

qRT-PCR Assay

All reverse transcriptase reactions were performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with both oligo-dT (Gene Link, Hawthorne, NY) and random hexamers (Roche) priming for a more complete transcription of all RNA including the fragmented RNA often found in paraffin-embedded specimens as previously described (Bostick, P.J., 1999; Miyashiro, I., 2001). The RealTime PCR assay was performed using iCycler iQ RealTime Thermocycler (4 color) Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR reaction mixture consisted of cDNA template from 250 ng of total RNA, 1 μM of each primer, 0.3 μM FRET probe, 1 U AmpliTaq Gold polymerase (Applied Biosystems, Branchburg, NJ), 200 μM of each dNTP, 4.5 mM MgCl2, 10 μg of bovine serum albumin, and 10X AmpliTaq Buffer to a final volume of 25 μl. Samples were amplified with a precycling hold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min for GAPDH, annealing at 58°C for MAGE-A3 (40 cycles), at 62°C for MART-1, at 62°C for GalNAc-T (42 cycles), at 65°C for Pax3, and extension at 72°C for 1 min. The following conditions may be used for other markers: 59°C for MITF; 62°C for TRP-2 (42 cycles); 58°C for tyrosinase; 55°C for C-MET; 54°C for stanniocalcin-1; 59°C for HSP 27; 55°C for cytokeratin 20; and 60°C for beta-HCG.

For ROC curve analysis, all reactions were performed at same conditions but 45 cycles. All PCR conditions and annealing temperature for each marker were optimized at initial experiments and ROC curve analysis.

Positive controls (melanoma cell lines and PE metastatic melanoma tissues), negative controls from tumor-free lymph nodes, and reagent controls (reagent alone without RNA or cDNA) for the qRT-PCR assays were included in each assay run. Each assay was performed at least three times to verify the results and the mean

copy number was used for analysis. If twice or more positive copy numbers were obtained in the same sample, the sample by the marker was considered to be positive. If only once positive copy number was detected in the three assays, the sample was thought to be negative. In the patient who have two or more identified SLNs, the highest copy number among them was listed as a chosen value.

The standard curve for quantifying mRNA copy number was established by amplifying nine aliquots of templates with known copy numbers (10^0 to 10^8 copies). Specific MAA cDNA was synthesized as follows; RT-PCR and sample RNA was performed, run on 2% agarose gel electrophoresis, and the cDNA was extracted using the QIAquick gel extraction method (Qiagen, Valencia, CA) according to the manufacturer's instructions. The MAA cDNA was ligated into pCR II-TOPO cloning vector (Invitrogen, San Diego, CA), the cDNA clones were transformed into *Escherichia coli* DH5-α cells, and cultures were expanded as previously described (Miyashiro, I., 2001). Plasmids containing the target gene were purified and quantified for use in the RealTime PCR setup. To confirm that the inserted PCR product size is correct, plasmids were digested with specific restriction enzymes, and the cDNA clone PCR products were then run on gel electrophoresis.

Example 6

Statistical Analysis

To investigate the association between single and multimarker combinations and clinicopathological parameters, Student's *t*-test was used for continuous variables. Chi-square test was used for categorical variables. Kappa analysis and Spearman correlation coefficient analysis were used for the comparison between the copy numbers of each mRNA marker. The cumulative disease-free survival rates for patient groups were calculated using the Kaplan-Meier methods and compared by using the log-rank test. Cox proportional hazard model was used for multivariate analysis of variables associated with the disease-free survival. All p values which were two-sided at a value of $\leqq 0.05$ were considered to be statistically significant.

Example 7

MAA Expression In Melanoma Cell Lines

MART-1, MAGE-A3, GalNAc-T, and PAX3 mRNA expressions were measured by qRT-PCR assay in ten melanoma cell lines. The PCR amplification of the serially diluted cDNA standard templates of each marker showed a logarithmic

signal increase (**Figure 1A,** representative qRT-PCR analysis for MART-1). The standard curve was generated by using the threshold cycle (Ct) of templates with known numbers of copies (**Figure 1B,** only for MART-1 is shown). The Ct of each sample was plotted on the standard curve, and the mRNA copy number was calculated by the iCycler iQ RealTime Detection System Software (Bio-Rad Laboratories). All four markers were expressed in every melanoma cell line.

The MART-1 mRNA copy number ranged from 5.66×10^3 to 4.56×10^7 copies (median 2.32×10^7 copies) per 250 ng of total RNA from 10 individual melanoma cell lines. The MAGE-A3 mRNA copy number ranged from 6.34×10^3 to 1.31×10^6 copies (median 7.61×10^5 copies), the GalNAc-T mRNA copy number ranged from 24 to 5.05×10^5 copies (median 3.58×10^4 copies), and the PAX3 mRNA copy levels were from 2.25×10^4 to 4.00×10^5 copies (median 2.12×10^5 copies). The house-keeping gene GAPDH mRNA copy number ranged from 3.19×10^7 to 1.56×10^8 copies (median 1.05×10^8 copies). The marker expressions and the size of PCR products were certified by a run on 2% agarose gel electrophoresis after qRT-PCR assay.

Example 8

ROC curves analysis

ROC curve analysis was performed to define the diagnostic accuracy of the MM qRT-PCR assay of the present invention for the detection of metastatic melanoma in PE specimens. Each marker mRNA copy level in 32 pathology-verified metastatic lesions from melanoma patients was significantly higher than that in 39 pathology-defined cancer negative lymph nodes. ROC curve analysis is based on a plot of sensitivity as a function of 1-specificity (Henderson, A.R., 1993; Mitas, M., 2001). **Figure 2** provides ROC curves for various markers.

Table 1 ROC curves analysis

Mark r	W (Area und r ROC curv) ± SE	S nsitivity	Specificity
MART-1	0.906 ± 0.039	0.813	1.000
MAGE-A3	0.903 ± 0.039	0.781	1.000
GalNAcT	0.813 ± 0.053	0.594	1.000
PAX3	0.968 ± 0.023	0.969	0.923

ROC curve analysis is the most commonly used statistical method for assessing the accuracy of diagnostic tests (Henderson, A.R., 1993; Mitas, M., 2001). The area under the ROC curve with a defined confidence interval is a measure of diagnostic accuracy such that values between 0.5 and 0.7 indicate low accuracy, values between 0.7 and 0.9 indicate moderate accuracy and values more than 0.9 indicate high accuracy.

In this study, the value of each marker was as follows; MART-1, W = 0.906; MAGE-A3, 0.903; GalNAc-T, 0.813; PAX3, 0.968 (**Table 1 and Figure 2**). The sensitivity and specificity for given cut-off points (optimized PCR cycle number) were also evaluated (**Table 1**). None of the MART-1, GalNAcT and MAGE-A3 mRNA copy levels were expressed under the assay's optimal conditions in normal lymph nodes (n = 39). Thus, the methods of the present invention, unlike conventional methods do not produce false-positive results. Only one normal lymph node expressed PAX3 mRNA, however, the copy number was one. The values in ROC curve analysis and the sensitivity and specificity of each marker were acceptable and feasible for detection of metastatic melanoma in PE specimens.

Example 9

Comparison Between Frozen and PE Specimens in the SLN Sample

The sensitivity of the MM qRT-PCR assay was further validated by comparing for each marker mRNA copy level of paraffin-embedded specimens with frozen sections in 31 SLNs (ten SLNs were pathology defined melanoma-positive lymph nodes, and 21 SLNs were histologically negative).

Table 2 Consistency of mRNA expression between paraffin-embedded and frozen SLNs

Marker	Consistency of results (%)	*Kappa* analysis			Spearman correlation coefficient	
		Coefficient	95% CI	p-value	Coefficient	p-value
MART-1	30/31 (97%)	0.912	0.744, 1.081	<0.0001	0.963	<0.0001
MAGE-A3	27/31 (87%)	0.597	0.260, 0.934	0.0022	0.868	<0.0001
GalNAc-T	27/31 (87%)	0.688	0.409, 0.968	0.0003	0.835	<0.0001
PAX3	30/31 (97%)	0.912	0.744, 1.081	<0.0001	0.970	<0.0001

Positivity of each marker in PE sections was highly coincided with that in corresponding frozen sections (**Table 2**). Moreover, Spearman correlation coefficient analysis and Kappa analysis revealed significant correlation in each marker mRNA copy level between PE sections and corresponding frozen sections (**Table 2**). Surprisingly, the study demonstrated not only a high coincidence of positivity but also significant correlation in each marker mRNA copy level between frozen and PE specimens. The study revealed that marker panels of the present invention can be used in both PE and frozen specimens.

Example 10

Dilution study

The sensitivity of mRNA marker detection by the qRT-PCR assay was verified by serial dilution analysis of total RNA from 1000 melanoma cells obtained from paraffin-embedded specimens using the LCM. The assays were repeated at least three times for each marker to verify the results and reduce false-positive and false-negative results. Representative results are shown as **Table 3**.

In several samples, MART-1, MAGE-A3, GalNAcT and PAX3 mRNA copy levels were detectable in 1/100 diluted total RNA which is approximately equal to total RNA from 10 melanoma cells in the qRT-PCR assay.

Dilution study using the LCM was performed to define the sensitivity for detection of PE occult melanoma cells in the assays. It is known that the mRNA levels for individual markers vary in individual melanoma cells because inter- and intra-tumor heterogeneity. Therefore the results may not always representative of all SLN specimens assessed in this study. However, the detection sensitivities obtained in the present invention (10 to 50 metastatic melanoma cells in 100-mm thick PE SLN) unexpectedly allow detection of occult melanoma cells in SLNs.

Table 3

Marker	Sample	Copy number at the diluted samples (No. of estimated melanoma cells)						
		1 (1000)	1/2 (500)	1/10 (100)	1/20 (50)	1/100 (10)	1/200 (5)	1/1000 (1)
MART-1	A	167	139	38	7	2	0	0
	B	39	31	3	2	0	0	0
	C	166	83	12	2	0	0	0
MAGE-A3	B	183	161	8	2	1	0	0
	C	243	157	31	6	1	0	0
	D	70	35	5	2	0	0	0
GalNAc-T	E	18	12	4	2	0	0	0
	F	19	8	6	2	1	0	0
	G	7	4	1	0	0	0	0
pax3	A	47	18	2	1	0	0	0
	C	74	33	5	3	1	0	0
	D	42	22	12	0	0	0	0

Example 11

MM qRT-PCR assay in PE SLNs

MART-1, MAGE-A3, GalNAcT, and PAX3 mRNA copy levels were assessed using the qRT-PCR assay on 308 SLNs obtained from 215 clinically early-stage melanoma patients who underwent SLND. Of the 53 patients with histopathologically proven melanoma cells (48 detected by H&E staining, seven by

IHC alone) in the SLN, MART-1, MAGE-A3, GalNAcT, and PAX3 mRNA markers were expressed in the SLNs of 38 (72%), 24 (45%), 34 (64%), and 37 (70%) patients, respectively (**Table 4**). Among the patients with histopathologically positive SLNs, 47 (89%) of 53 expressed at least one detectable mRNA marker, and 20 patients (38%) demonstrated all four marker mRNA expression (**Table 4**). On the other hand, MART-1, MAGE-A3, GalNAcT, and PAX3 marker expression were detected from 162 patients with histopathologically proven melanoma-free SLNs (no evidence of tumor cells in SLN by H&E staining or IHC) as follows: 10 (6%), 8 (5%), 27 (17%), 28 (17%), respectively. Positivity of each mRNA marker in histopathologically positive SLNs was significantly higher than that in histopathologically negative SLNs (**Table 4**). Among 162 patients with histopathologically negative SLNs forty-eight patients (30%) had at least one melanoma marker detected and 19 patients (12%) had two or three markers expressed. No patients with histopathologically negative SLNs demonstrated all four marker expression.

Each mRNA copy number per 250 ng of total RNA in SLN from 215 patients ranged as follows: MART-1, 0 to 547,400 mRNA copies (mean ± SD: 5,926 ± 45,876 copies); MAGE-A3, 0 to 1,980 copies (28 ± 160 copies); GalNAc-T, 0 to 13,710 copies (88 ± 938 copies); Pax3, 0 to 11,060 copies (190 ± 917 copies). Mean copy level for each mRNA marker in histopathologically positive SLNs was significantly higher than that in histopathologically negative SLNs (**Table 4**). Patients' age, Breslow's thickness, and Clark level were also significantly different between histopathologically positive and negative SLN patients, respectively (**Table 4**). The study may suggest that mRNA marker copy labels by the qRT-PCR assays of the present invention are generally reflected by tumor volume or number of melanoma cells in SLN in spite of the tumor heterogeneity.

Spearman correlation coefficient analysis also showed a significant correlation ($p<0.001$) in comparison of MART-1 vs. MAGE-A3, MART-1 vs. GalNAcT, MART-1 vs. PAX3, MAGE-A3 vs. GalNAcT, MAGE-A3 vs. PAX3, and GalNAcT vs. PAX3 with the correlation coefficient 0.560, 0.488, 0.514, 0.526, 0.506, and 0.465, respectively. All SLN specimens were positive for GAPDH showing high integrity of the mRNA extracted from the tumor specimens. The

GAPDH mRNA copy number ranged from 1.02 x 10^3 to 7.24 x 10^6 copies (median 3.24 x 10^4 copies).

Tabl 4 Correlation of mRNA Markers with Histopath l gy Stat s f the SLNs

Risk Factor	H&E/IHC(+) SLN (n = 53)	H&E/IHC(-) SLN (n = 162)	p value
Age (mean +/- SD)	46.5	52.3	0.02
Gender: Male	30	97	
Female	23	65	0.8
Primary Site			
Head/neck	5	26	
Trunk	20	66	
Extremity	28	70	0.35
Breslow thickness (mm)			
<=1.00	6	55	
1.01-2.00	20	65	
2.01-4.00	19	26	
>4.00	7	13	0.001
Mean +/- SD(mm)	2.86 +/- 2.6	1.9 +/- 1.8	0.003
Clark level			
1	0	2	
2	4	11	
3	10	65	
4	35	72	
5	3	8	0.03
MART-1 expression			
Positive	15	152	
Negative	38	10	<0.0001
MART-1 copy number (Mean +/- SD)	24029 +/- 90652	3.4 +/- 27	0.0008
GalNAc-T expression			
Positive	19	135	
Negative	34	27	<0.0001
GalNac-T copy number (Mean +/- SD)	331 +/- 1882	7.9 +/- 29	0.03
Pax3 expression			
Positive	16	134	
Negative	37	28	<0.0001
Pax3 copy number (Mean +/- SD)	701 +/- 1734	22.7 +/- 177	<0.0001
MAGE-A3 expression			
Positive	29	154	
Negative	24	8	<0.0001
MAGE-A3 copy number (Mean +/- SD)	114 +/- 309	0.2 +/- 1	<0.0001
Number of mRNA marker			
0	6	114	
1	9	29	
2	10	13	
3	8	6	
4	20	0	<0.0001

In a median follow-up period of 60.4 months, thirty-one (58%) of 53 patietns with histopathologically positive SLNs and 39 (24%) of 162 patients with

histopathologically negative SLNs have recurred after SLND. The disease free survival (DFS) rate for 53 patients with histopathologically positive SLNs was significantly lower than for 162 patients with histopathologically negative SLNs (Log-rank test $p<0.0001$; **Figure 3, Table 4**). Among the 162 patients with histopathologically negative SLNs, only 12 (11%) of the 114 patients with SLNs that were with histopathologically negative and had no mRNA marker expressed had recurrence of disease. Among the 48 patients with SLNs that were histopathologically negative SLNs but which expressed one or more mRNA markers, 56% (27 of 48) had recurrence. A significantly worse DFS was associated with patients whose histopathologically negative SLN expressed one or more mRNA markers as compared to those patients with no mRNA marker present ($p<0.0001$) (**Table 5**). In particular, the increased number of mRNA marker expression significantly correlated with worse DFS rate among histopathologically negative SLN patients (**Figure 4A**). There have been 12 (41%) recurrences in 29 patients with one mRNA marker expressed, 10 (71%) recurrences in 14 patients with two markers expressed, and 5 (100%) recurrences in 5 patients with three marker expression (**Figure 4b**).

By multivariate analysis using Cox proportional hazard model, histopathologically negative SLN patients at increased risk of recurence had the combination of the MM expression (RR 3.26, 95% CI: 2.4-4.4, $p<0.0001$) and Breslow's thickness (RR 1.21, 95% CI: 1.1-1.4, $p=0.01$). In histopathologically negative SLN patients, the mRNA copy number of each marker in patients who recurred was significantly higher than that in patients who did not recur ($p<0.001$; **Table 6**). Among the total 215 patients, only MAGE-A3 mRNA copy levels correlated with disease recurrence (mean copy number in the patients with recurrence vs. no recurrence; 64 ± 265 vs 11 ± 59; $p=0.02$).

Table 5 Prognostic Significance of mRNA Marker Detection in the SLN versus Disease Recurrence

Risk Factor	No recurrenc (n = 123)	Recurr nc (n = 39)	p valu
Age (mean +/- SD)	51.9 +/- 16.3	53.5 +/- 15	0.58
Gender: Male	72	25	
Female	51	14	0.53
Primary Site			
Head/neck	15	11	
Trunk	57	9	
Extremity	51	19	0.011
Breslow thickness (mm)			
T1 <=1.00	48	10	
T2 1.01-2.00	52	13	
T3 2.01-4.00	14	12	
T4 <4.00	9	4	0.025
Mean +/- SD(mm)	1.7 +/- 1.6	2.4 +/- 2.0	0.034
Clark level			
1	2	0	
2	7	4	
3	54	11	
4	52	20	
5	6	2	0.41
AJCC stage			
I (T1 and N0)	48	10	
II (T2 or T3 or T4 and N0)	75	28	
III (Any T and N1/2)	0	0	0.13
MART-1 expression			
Positive	3	7	
Negative	120	32	<0.0005
MART-1 copy number (Mean +/- SD)	44.8 +/- 309	16904 +/- 76801	0.01
GalNAc-T expression			
Positive	8	19	
Negative	115	20	<0.0001
GalNac-T copy number (Mean +/- SD)	17 +/- 63	219 +/- 1585	0.13
Pax3 expression			
Positive	13	15	
Negative	110	24	<0.00006
Pax3 copy number (Mean +/- SD)	61.7 +/- 384	429 +/- 1436	<0.005
MAGE-A3 expression			
Positive	1	7	
Negative	122	32	<0.00002
MAGE-A3 copy number (Mean +/- SD)	6.7 +/- 42	68.5 +/- 261	<0.007
Number of mRNA marker			
0	102	12	
1	17	12	
2	4	9	
3	0	6	
4	0	0	<0.0001

Table 6 C x Pr p rtional Hazards Model of Multi Marker RT-PCR

	RR	Pvalue
Age (<60,>60)	1.47	0.24
Gender	1.15	0.42
Breslow	1.15	0.037
Clark	1.57	0.0037
Ulceration	2.4	0.13
Site (long rank test)		0.007
Tyr	6.7	<0.001
Mart	10.1	<0.001
Trp	5.57	<0.001
Galnac	7.66	<0.001
Mitf	6.03	<0.001
Pax3	4.66	<0.001
Mage	7.21	<0.001
Total 1 (log rank test)		<0.001
Total 1 (0,1,...,7)	1.8	<0.001
Total 2 (0,1,2+)	4.46	<0.001
Total 3 (0-1,2+)	15.7	<0.001

Table 6 gives the results of the univariate CoxPH analyses in the SN negative group. The markers are dichotomized as +/- expression. Total 1 is the number of positive markers. Total 2 uses two categories 0 positives, 1 positive or 2+ positive markers. Total 3 compares 0-1 positive markers vs 2+ positive markers. Two factors, site and total 1 employ the log rank test meaning that the variable was treated as a factor and we cannot compute an associated relative risk (RR).

The correlation of MM expression by qRT-PCR with patient's survival was also assessed. The survival rate for 53 patients with histopathologically positive SLNs was significantly lower than for 162 patients with histopathologically negative SLNs ($p<0.0001$). Among the 162 patients with histopathologically negative SLNs, the survival rate for patients with one or more mRNA marker expression was significantly lower than that for patients with no mRNA marker expression ($p<0.0001$; **Figure 5**). Furthermore the patient's worse survival correlated with the increased number of mRNA marker expression similar to the results of DFS analysis ($p<0.0001$).

The present invention may be embodied in other specific forms without departing from its essential characteristics. The described embodiment is to be considered in all respects only as illustrative and not as restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of the equivalence of the claims are to be embraced within their scope.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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